THE INCORPORATION OF RADIOACTIVE PHOSPHORUS IN THE INFLUENZA VIRUS AND ITS DISTRIBUTION IN SEROLOGICALLY ACTIVE VIRUS FRACTIONS

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Graham & McLelland (1949, 1950) showed that when the PR 8 strain of influenza virus A was grown in fertile eggs, into the allantoic sacs of which radioactive inorganic phosphate had been introduced, the virus incorporated ^{32}P into its structure. Graham (1950) showed that the incorporated ^{32}P was to be found partly in the phospholipid and partly in the nucleic acid fractions of the virus.

This paper describes the production of influenza virus labelled with ^{32}P, and a study of the distribution of the isotope in serologically active fractions of the virus produced by ether disintegration.

PRODUCTION OF INFLUENZA VIRUS LABELLED WITH ^{32}P

In the original work of Graham & McLelland (1949) the virus was grown in eggs inoculated with less than 1 μc. of ^{32}P, since it was thought that larger amounts of ^{32}P might be lethal to the embryo. Later Graham, Dempster & Buchner (1952) found that this was not so and that much larger amounts of ^{32}P could be used. We ourselves have noted no deaths in eggs inoculated by the allantoic or yolk-sac routes with 100–500 μc. ^{32}P.

A major difficulty in the production of ^{32}P labelled virus lies in the separation of the virus from contaminating radioactive phosphate in the infected allantoic fluids, and much preliminary work was necessary to determine the best method of virus purification. Preparations were regularly obtained in which the non-viral radioactivity amounted to less than 10% of that carried by the virus by the following technique. The D.S.P. strain of influenza virus A was used throughout the work. Radioactive phosphorus was obtained from the Atomic Energy Research Establishment at Harwell in the form of carrier-free orthophosphoric acid, this was neutralized with sodium hydroxide, diluted to contain 100 μc. in 0·2 ml. and sterilized by immersion in boiling-water for 15 min. The radioactive phosphate solution was introduced into the allantoic sac of sixteen 12-day-old fertile eggs in a dose of 0·2 ml., each egg thus receiving 100 μc. of ^{32}P. After 4 hr. incubation at 36° C. the eggs were inoculated by the allantoic route with 0·1 ml. of a 1:100 dilution of D.S.P. virus-infected allantoic fluid. After a further 40 hr. incubation the eggs were opened, the chorio-allantoic membranes torn through and allowed to bleed into the allantoic fluid. The blood-stained allantoic fluid was collected in a receiver chilled with ice. From the 160 ml. of fluid collected the red cells carrying

the adsorbed virus were sedimented by centrifugation and washed twice with 160 ml. of ice-cold saline. The adsorbed virus was then eluted from the cells into 16 ml. of saline by 4 hr. incubation at 37° C. The cells were then sedimented by centrifugation. The supernatant fluid, representing a tenfold concentration of the virus in the original infected allantoic fluid, still contained a large amount of non-viral ^{32}P, and further purification was therefore necessary. The fluid was adsorbed with 10% guinea-pig red cells, the cells were then separated by centrifugation and washed six times with 25 ml. quantities of ice-cold saline. After the final washing the cells were suspended in 8 ml. of saline containing 1 in 10,000 $CaCl_2$ and were incubated for 4 hr. at 37° C. to allow elution of the virus. The cells were then sedimented by centrifugation and removed and 0·08% sodium azide was added to the supernatant fluid. This constituted the labelled virus preparation. A 1 ml. sample of the preparation was adsorbed twice with 10% guinea-pig red cells, and the radioactivity of the original and adsorbed fluids was tested. The red-cell agglutinin and complement-fixing antigen titres were also measured.

TECHNIQUE OF RADIOACTIVITY MEASUREMENTS

1 ml. samples of the fluids to be tested were placed in shallow metal dishes 2·5 cm. in diameter, the fluid thus giving a layer 1·6 mm. thick. The dishes were then placed beneath a Geiger-Muller counter of the end-window type at a distance of 2 cm. Counting periods ranged from 1 to 5 min. according to the potency of the preparation. Under these conditions 100 μc. of ^{32}P would register 3,650,000 counts in 1 min. (c.p.m.). The background count averaged 8 c.p.m.

RESULTS

The labelled virus preparation had a red-cell agglutinin titre of 16,000 and a complement-fixing antigen titre of 48. Two successive adsorptions with 10% red cells reduced both titres to zero. The original preparation registered 913 c.p.m. in the Geiger counter, while the adsorbed fluid registered only 36 c.p.m. It was therefore concluded that 877 c.p.m. represented ^{32}P incorporated in the virus. When the virus was eluted from the red cells used in the adsorption the eluate registered 845 c.p.m. Table 1 shows the results of five such experiments in which the virus was effectively labelled.

Table 1. *Radioactivity of labelled virus preparations before and after red-cell adsorption*

Preparation	Red-cell agglutinin titre	Complement-fixing antigen titre	Geiger count per min. per ml.	Geiger count after red-cell adsorption	Virus radio-activity (counts/min./ml.)
1	8,192	14	610	49	561
2	16,384	48	913	36	877
3	48,000	48	2,563	66	2497
4	8,192	12	336	28	308
5	25,600	24	732	35	697

ETHER FRACTIONATION OF LABELLED VIRUS

Hoyle (1950, 1952) showed that influenza virus particles could be disintegrated by treatment with ether with the liberation of separate red-cell agglutinating and complement-fixing 'soluble antigen' particles of smaller size. It was suggested that the virus elementary body consisted of an aggregate of red-cell agglutinating and complement-fixing particles enclosed in a lipid envelope. Ether destroys this envelope and releases the smaller units. Prolonged shaking with ether, however, causes denaturation of the virus protein with loss of its serological properties. When the ether fractionation technique was applied to the labelled virus preparations it was found that the duration of the ether treatment, and especially the amount of shaking, was much more critical than had been found in previous work, this being possibly due to the very great purity of the labelled preparations resulting from the double cycle of adsorption-elution and the numerous washings necessary to remove inorganic phosphate. Satisfactory ether fractionation was attained in three out of five experiments. In one experiment the ether treatment proved inadequate and only slight separation of agglutinin and soluble antigen occurred, while in another experiment the ether treatment was excessive and the serological properties were destroyed.

The technique used in the three successful experiments was the following one. The labelled virus preparation was shaken for 5 sec. with half its volume of ether and incubated for 1 hr. at 37° C. in a closed tube. It was again shaken for 5 sec. and incubated for a further hour. The tube was then centrifuged and the ether layer removed. A slight deposit of denatured protein resulted from the ether treatment. Preliminary work had shown that this deposit contained phospholipid which could be extracted by ethanol. The denatured protein was therefore washed with saline and extracted overnight with ethanol. The ethanol extract was then added to the ether, the whole constituting the *phospholipid fraction.* The residual denatured protein was dissolved in N/1 sodium hydroxide and neutralized; this is referred to as the *denatured protein fraction.*

The ether-treated virus preparation was incubated in an open tube overnight to remove all traces of ether. After removal of a sample for serological examination and radioactivity measurements the remainder was adsorbed with 20 % guinea-pig red cells, and centrifuged. The cells carrying the adsorbed haemagglutinin were washed twice with ice-cold saline and eluted for 4 hr. at 37° C. into saline containing 1 in 10,000 $CaCl_2$ and 50 Australian units of crystalline receptor destroying enzyme (R.D.E.) per ml. The R.D.E. was added to ensure complete recovery of the haemagglutinin. The cells were then removed by centrifugation and the supernatant fluid constituted the *red-cell agglutinin fraction.*

The supernatant of the ether-treated suspension after red-cell adsorption was again adsorbed with red cells to ensure complete removal of haemagglutinin, and after removal of the cells constituted the complement-fixing *soluble antigen fraction.*

The radioactivity of the various fractions was then measured and the serological titres determined. Red-cell agglutinin was measured by the Salk (1944) test,

0·4 ml. of a range of dilutions of the preparation being mixed with 0·4 ml. of 1:250 guinea-pig red cells. Readings were made after 2 hr. at room temperature. Complement-fixing antigen was measured by the technique of Hoyle (1945), using pooled human convalescent serum from the A prime influenza epidemic of 1951. This serum reacted mainly with the group antigen of the virus, the serum containing very little antibody to the specific antigen of the D.S.P. strain of influenza virus.

Results of a typical experiment are shown in Table 2. The original labelled virus preparation had a Salk titre of 16,384 and a complement-fixing antigen titre of 48. It registered 913 c.p.m. in the Geiger counter. Adsorption with red cells reduced the serological titre to zero and the Geiger count to 36 c.p.m. Therefore 877 c.p.m. represented virus ^{32}P and 36 c.p.m. contaminating inorganic ^{32}P.

Table 2. *Ether fractionation of the* D.S.P. *strain of influenza virus labelled with* ^{32}P

Material	Red-cell agglutinin titre	Complement-fixing antigen titre	Geiger count per min. per ml.
Original labelled virus preparation	16,384	48	913
Preparation after two adsorptions with red cells	Nil	Nil	36
Ether-treated suspension	32,768	72	708
Phospholipid fraction (ethereal extract + ethanol extract of denatured protein)	—	—	160
Residual denatured protein fraction	—	—	42
Red-cell agglutinin fraction	32,768	8	65
Complement-fixing soluble antigen fraction	Nil	64	646

On treatment with ether the agglutinin titre was doubled and the complement-fixing antigen titre was also slightly increased. The ether-treated preparation registered 708 c.p.m. The phospholipid fraction registered 160 c.p.m. and the denatured protein 42 c.p.m. Adsorption of the ether-treated suspension with red cells resulted in almost complete separation of red-cell agglutinin and complement-fixing antigen. The soluble antigen fraction contained no haemagglutinin. It gave a complement-fixing antigen titre of 64 and registered 646 c.p.m. in the Geiger counter. The red-cell agglutinin fraction had a haemagglutinin titre of 32,768. It contained a little complement-fixing antigen, giving a titre of 8, and its radioactivity was 65 c.p.m. It was evident that the radioactivity of the ether-treated suspension was associated with the complement-fixing soluble antigen and not with the haemagglutinin, the slight radioactivity of the haemagglutinin fraction being entirely accountable by its small content of complement-fixing antigen.

PHOSPHOLIPID CONTENT OF THE VIRUS

Previous work (Hoyle, 1952) had suggested that the virus elementary body was enclosed by a lipid envelope derived from the wall of the host cell. When virus preparations are shaken with ether a precipitate of denatured protein occurs at the ether-water interface. Lipid is present in the ether extract, but this does not

represent the whole of the virus lipid since more can be extracted from the denatured protein precipitate with ethanol. It seems probable that the lipid is present as a lipoprotein which is denatured by ether treatment, the lipid being partly dissolved by the ether and partly remaining attached to the denatured protein. In one experiment with labelled virus the ether extract registered 33 c.p.m. while an ethanol extract of the denatured protein registered 127 c.p.m. In three experiments in which ether disintegration of the virus was achieved without serious loss of serological properties the combined lipid extracts accounted for 18, 23 and 23 % of the total virus radioactivity. By prolonged shaking with ether it is possible to denature the whole of the virus protein with total loss of serological properties, and in one experiment in which this was done the radioactivity of the ether extract and of an ethanol extract of the denatured protein represented 26 % of the total virus radioactivity. It was, therefore, concluded that some 20–25 % of the radioactivity of ^{32}P labelled virus preparations is due to phospholipid. This result is in agreement with that of Graham (1950), who showed that the radioactivity of ^{32}P labelled virus was partly due to nucleic acid and partly to phospholipid, the nucleic acid fraction being about 4 times as active as the lipid fraction.

PROGRESSIVE DENATURATION OF THE VIRUS PROTEIN BY ETHER

The radioactivity of the denatured protein precipitate resulting from ether treatment is not entirely due to lipid. Even after prolonged extraction with ethanol the residual precipitate is still radioactive. In two experiments in which there was no apparent loss of serological properties this residual radioactivity was 42 and 44 c.p.m., representing 4·9 and 6·3 % of the total virus radioactivity. In an experiment in which ether treatment resulted in 50 % loss of serological properties the residual denatured protein gave a count of 140 c.p.m., representing 23 % of the total. When the virus protein was totally denatured by ether treatment the residual denatured protein after ethanol extraction gave a count of 1504 c.p.m., representing 60 % of the total virus radioactivity.

Since the non-lipid virus radioactivity is almost certainly due to nucleic acid it is probable that the radioactivity of the residual denatured protein is due to nucleic acid and this radioactivity affords some measure of the amount of denaturation of the virus nucleoprotein which occurs on ether treatment. It is of interest that when the virus nucleoprotein is completely denatured by ether treatment the whole of the nucleic acid is not split off; a large part remains combined with the denatured protein. Thus in the experiment in which complete loss of serological properties resulted from ether treatment the non-lipid radioactivity amounted to 1980 c.p.m., of which 1504 c.p.m. was found in the denatured protein precipitate, while 476 c.p.m. was found in the aqueous phase after ether treatment, presumably as free nucleic acid.

DISTRIBUTION OF THE VIRUS NUCLEIC ACID

The radioactivity of ether-treated virus suspensions is in part due to contaminating inorganic ^{32}P present in the original labelled virus preparation, but the major part of the activity must in view of the results of Graham (1950) be attributed to nucleic

acid. About 75% of the original virus radioactivity appears in the ether-treated suspension. When the suspension is adsorbed with red cells the major part of the activity appears in the complement-fixing soluble antigen fraction. The small amount of activity present in the agglutinin fraction is exactly related to the complement-fixing antigen content of this fraction. The results of three experiments are shown in Table 3. They indicate that the radioactivity is associated with the complement-fixing soluble antigen and not with the haemagglutinin. The haemagglutinin does not contain ^{32}P and is evidently not a nucleoprotein.

Table 3. *Distribution of virus nucleic acid radioactivity*

Expt.	Material	Haemagglutinin titre	Complement-fixing antigen titre	Geiger count per min. per ml.	Corrected Geiger count per min. per ml.
1	Ether-treated suspension	32,768	72	708	672
	Agglutinin fraction	32,768	8	65	65
	Soluble antigen fraction	Nil	64	646	610
2	Ether-treated suspension	51,200	24	486	451
	Agglutinin fraction	51,200	2·5	51	51
	Soluble antigen fraction	Nil	24	454	418
3	Ether-treated suspension	8,192	7	355	306
	Agglutinin fraction	8,192	Nil	2	2
	Soluble antigen fraction	Nil	7	357	308

Note. (1) The corrected Geiger count is the true virus non-lipid radioactivity obtained by subtracting from the observed radioactivity the activity due to contaminating inorganic ^{32}P present in the original virus preparation. This activity appears in the ether-treated suspension and in the soluble antigen fraction but not in the haemagglutinin fraction. (2) It will be noted that in each of the three experiments the sum of the radioactivities of the agglutinin and soluble antigen fractions is slightly greater than that of the original ether-treated suspension. This is probably a sampling error, but might be due to slight differences in the self-screening properties of the various preparations.

RADIOACTIVITY OF THE SOLUBLE ANTIGEN

The major part of the non-lipid radioactivity of the virus appears in the soluble antigen fraction, and it seems probable that the antigen is a nucleoprotein. However, it was thought possible that the effect of ether treatment might be to split nucleic acid from the virus protein, and that the radioactivity of the soluble antigen fraction might be due to free nucleic acid which was not actually combined with the antigen. The following experiments indicate that this is not so and that the antigen is itself radioactive.

Previous work had shown that the antigen could be precipitated by half saturation with ammonium sulphate. Considerable technical difficulty was experienced in precipitating the antigen from the radioactive preparations as a result of their great purity. Addition of ammonium sulphate resulted in the appearance of only a faint opalescence and it proved extremely difficult to centrifuge down the precipitate with the result that great losses of antigen occurred. However, the experiment was done and the slight precipitate obtained was washed with half

saturated ammonium sulphate and redissolved in water. Serological and radioactivity measurements gave the following result:

Original soluble antigen fraction	Complement-fixing antigen titre, 64 Geiger count, 610 c.p.m.
Redissolved ammonium sulphate precipitate	Complement-fixing antigen titre, 14 Geiger count, 101 c.p.m.

It appeared that the antigen was in fact radioactive. In a second experiment the antigen was subjected to purification by six serial precipitations with ammonium sulphate. The final product had a complement-fixing antigen titre of 3·0 and registered 21 c.p.m. in the Geiger counter.

Soluble antigen can also be precipitated with lanthanum acetate and partially recovered from the precipitate by extraction with sodium phosphate. A radioactive soluble antigen fraction was precipitated with an equal volume of 1% lanthanum acetate, the precipitate washed with water and extracted with 2·5% disodium phosphate solution with the following result:

Original soluble antigen fraction	Complement-fixing antigen titre, 32 Geiger count, 244 c.p.m.
Phosphate extract of lanthanum acetate precipitate	Complement-fixing antigen titre, 10 Geiger count, 87 c.p.m.

These experiments indicate that the radioactivity of the soluble antigen fraction is due to the antigen, and that the antigen must therefore contain ^{32}P.

DISCUSSION

This work shows that when influenza virus is grown in fertile eggs in which radioactive phosphate has been introduced, the virus incorporates ^{32}P into its structure. This confirms the original work of Graham & McLelland (1949). It had been hoped that sufficient ^{32}P might be introduced into the virus to enable studies to be made of the fate of radioactive virus introduced as a primary inoculum in fertile eggs. The amount of ^{32}P incorporated in the virus is, however, probably too small for this purpose. The labelled virus preparations obtained in this work had an average haemagglutinin titre of 21,000, and an average radioactivity of 820 c.p.m./ml. Since the maximum amount of virus which can be induced to enter the cells of the chorio-allantoic membrane in growth cycle experiments is from 500 to 1000 haemagglutinin units the radioactivity of such a primary inoculum would be only 20–40 c.p.m. This amount is probably inadequate for reliable results. It is, however, possible that the virus might be more effectively labelled by the use of larger amounts of ^{32}P, and also that the sensitiveness of the counting technique could be increased above that attained in the present work.

In our experiments 100 μc. of ^{32}P registered 3,650,000 c.p.m. This quantity of ^{32}P should actually emit 3,700,000 β particles per second, hence the counting efficiency was only about 1·7%. By the use of dried preparations closely applied to the window of the Geiger counter, Graham & McLelland (1950) attained a much higher counting efficiency. However, our virus preparations were much more effectively labelled than those of Graham & McLelland who calculated that in their

preparations there was only one atom of ^{32}P in 66,500 virus particles. The following calculation indicates the presence in our preparations of one atom of ^{32}P in about 670 virus particles. 100 μc. of ^{32}P containing $6{\cdot}6 \times 10^{12}$ atoms registered 3,650,000 c.p.m. by our technique. Hence 1 ml. of our average virus preparation registering 820 c.p.m. contained 1482×10^6 atoms of ^{32}P. The average haemagglutinin titre of our preparations was 21,000. Since 15 million red cells were used in the haemagglutination test and since it requires 3–4 virus particles per cell to give full agglutination, the preparation must have contained about 10^{12} particles per ml. This gives a ratio of one atom of ^{32}P to about 670 virus particles. Our preparations therefore contained about 100 times as much ^{32}P as those of Graham & McLelland. This difference is due to the use in our work of a dose of ^{32}P per egg 500 times as large as that used by them.

About 20–25 % of the ^{32}P in the virus appears to be present as phospholipid, a result confirming that of Graham (1950). Although no attempt has been made in this work to determine directly the nature of the non-lipid ^{32}P, it is undoubtedly linked to the virus protein and in view of the results of Graham (1950) there can be little doubt that it is present as nucleic acid. When the virus preparation is treated with ether most of the non-lipid ^{32}P remains in the ether-treated suspension, but a varying amount is found in the denatured protein precipitate which results from ether treatment. The results suggest that shaking with ether causes a progressive denaturation of virus protein, the lipoprotein being attacked first, and the nucleoprotein later. It would be expected that the lipoprotein would be denatured first if it is present as a surface envelope. Prolonged shaking with ether causes total denaturation of the virus protein with loss of all serological properties, but by careful control of the extent of the ether treatment it is possible to denature the virus lipoprotein and disintegrate the particle without serious loss of serological properties. When this is done most of the non-lipid ^{32}P is found in the aqueous phase of the ether-treated suspension. Adsorption of the ether-treated suspension with red cells results in more or less complete separation of red-cell agglutinin and complement-fixing soluble antigen. Soluble antigen fractions can be produced which contain no haemagglutinin at all. Haemagglutinin fractions usually contain small amounts of complement-fixing antigen. The non-lipid ^{32}P is almost entirely found in the soluble antigen fraction. Such radioactivity as is found in the haemagglutinin fraction is exactly related to the complement-fixing antigen titre of the fraction. The whole of the non-lipid ^{32}P of the virus appears to be present in the complement-fixing soluble antigen, the haemagglutinin apparently containing no phosphorus.

Hoyle (1952) brought evidence to suggest that the soluble antigen was a nucleoprotein, while the haemagglutinin was an enzymically active protein which was probably nucleic acid free. The present results confirm this conclusion, and afford strong evidence in favour of the hypothesis that the complement-fixing soluble antigen is the fundamental replicating nucleoprotein of the influenza virus.

SUMMARY

1. When the D.S.P. strain of influenza virus A is grown in eggs into which 100 μc. of radioactive inorganic phosphate has been introduced the virus incorporates ^{32}P into its structure.

2. Some 20–25% of the virus ^{32}P is found in the virus phospholipid; the remainder is combined with the virus protein and is probably present in the virus nucleic acid.

3. When the virus is disintegrated by ether treatment with the liberation of separate red-cell agglutinating and complement-fixing 'soluble antigen' particles the non-lipid ^{32}P is found to be associated with the soluble antigen fraction and not with the haemagglutinin.

4. It is suggested that the complement-fixing soluble antigen is a nucleoprotein while the haemagglutinin is a phosphorus-free protein.

REFERENCES

GRAHAM, A. F. (1950). The chemical analysis of purified influenza virus A (PR8 strain) containing radioactive phosphorus. *Canad. J. Res.* **28**, 186.

GRAHAM, A. F., DEMPSTER, G. & BUCHNER, B. (1952). The toxicity of P^{32} for normal and influenza virus-infected embryos. *J. Bact.* **63**, 426.

GRAHAM, A. F. & MCLELLAND, L. (1949). Uptake of radioactive phosphorus by influenza virus. *Nature, Lond.*, **163**, 949.

GRAHAM, A. F. & MCLELLAND, L. (1950). The uptake of radioactive phosphorus by influenza virus A (PR8 strain). *Canad. J. Res.* **28**, 121.

HOYLE, L. (1945). An analysis of the complement-fixation reaction in influenza. *J. Hyg., Camb.*, **44**, 170.

HOYLE, L. (1950). The multiplication of influenza viruses in the fertile egg. *J. Hyg., Camb.*, **48**, 277.

HOYLE, L. (1952). Structure of the influenza virus. *J. Hyg., Camb.*, **50**, 229.

SALK, J. E. (1944). A simplified procedure for titrating haemagglutinating capacity of in fluenza virus and the corresponding antibody. *J. Immunol.* **49**, 87.

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